Supplemental Information

Perivascular Gli1^{+} progenitors are key contributors to injury-induced organ fibrosis

Rafael Kramann, Rebekka K. Schneider, Derek P. DiRocco, Flavia Machado, Susanne Fleig, Philip A. Bondzie, Joel M. Henderson, Benjamin Ebert and Benjamin D. Humphreys

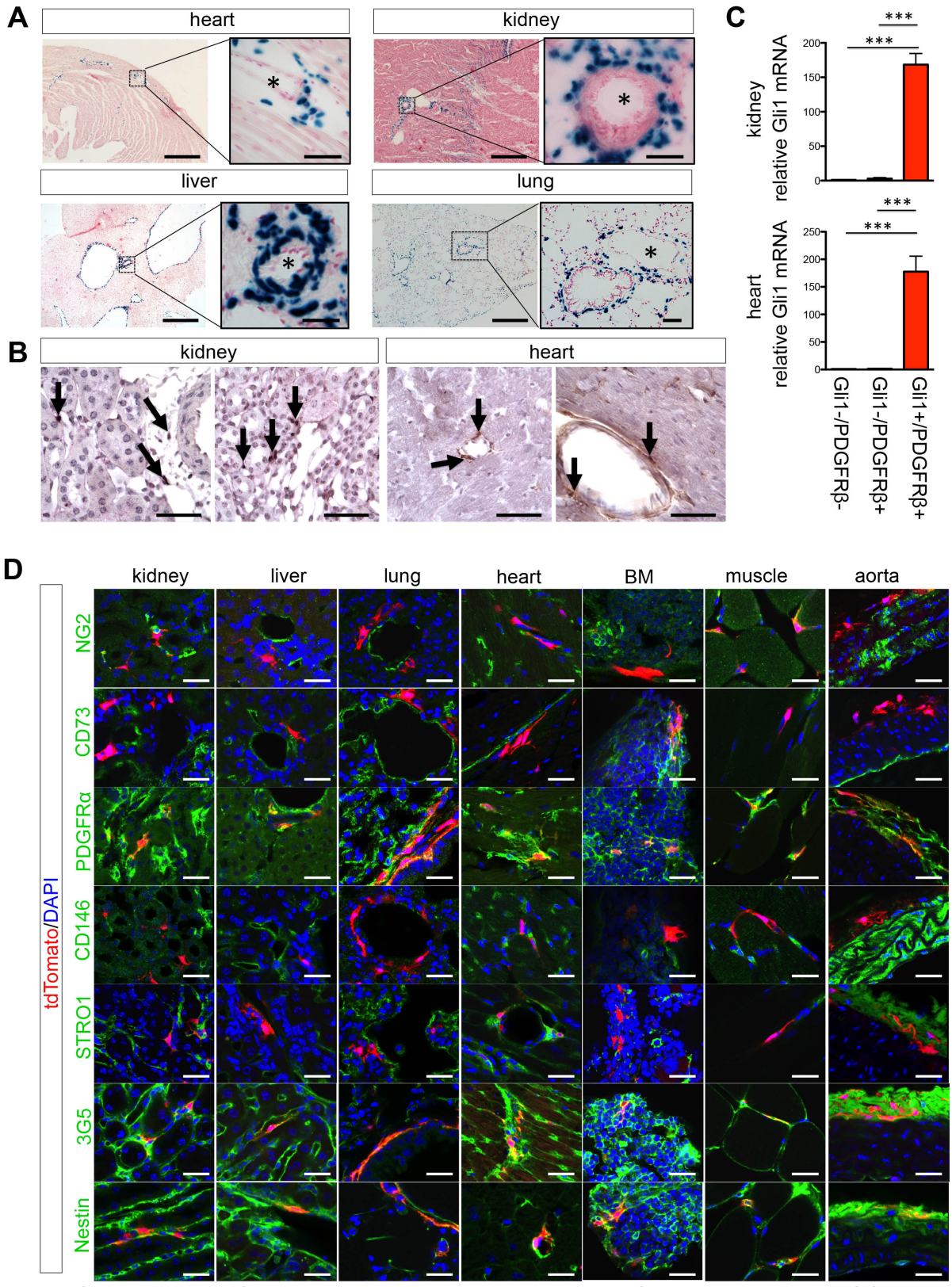

Figure S1: Related to figure 1. Perivascular cells express Gli1 and a typical MSC surface marker pattern.

(A) X-gal stained tissue sections from the Gli1-nLacZ mouse indicating Gli1 expressing cells are specifically located around the vasculature (* arteries). Scale bars: 500µm, inserts 50µm

(B) Immunohistochemical staining for endogenous Gli1 in mouse kidney and heart. Gli1+ cells (arrows) were located in the interstitium and adventitia of arteries. Scale bars 50µm

(C) Relative Gli1 mRNA expression of sorted cell populations of bigenic Gli1CreERt^2; tdTomato mice from kidney and heart. ***p<0.001 by one way ANOVA with posthoc Tukey

(D) Immunostaining for reported pericyte and MSC markers in healthy adult tissues. Scale bars: 25µm

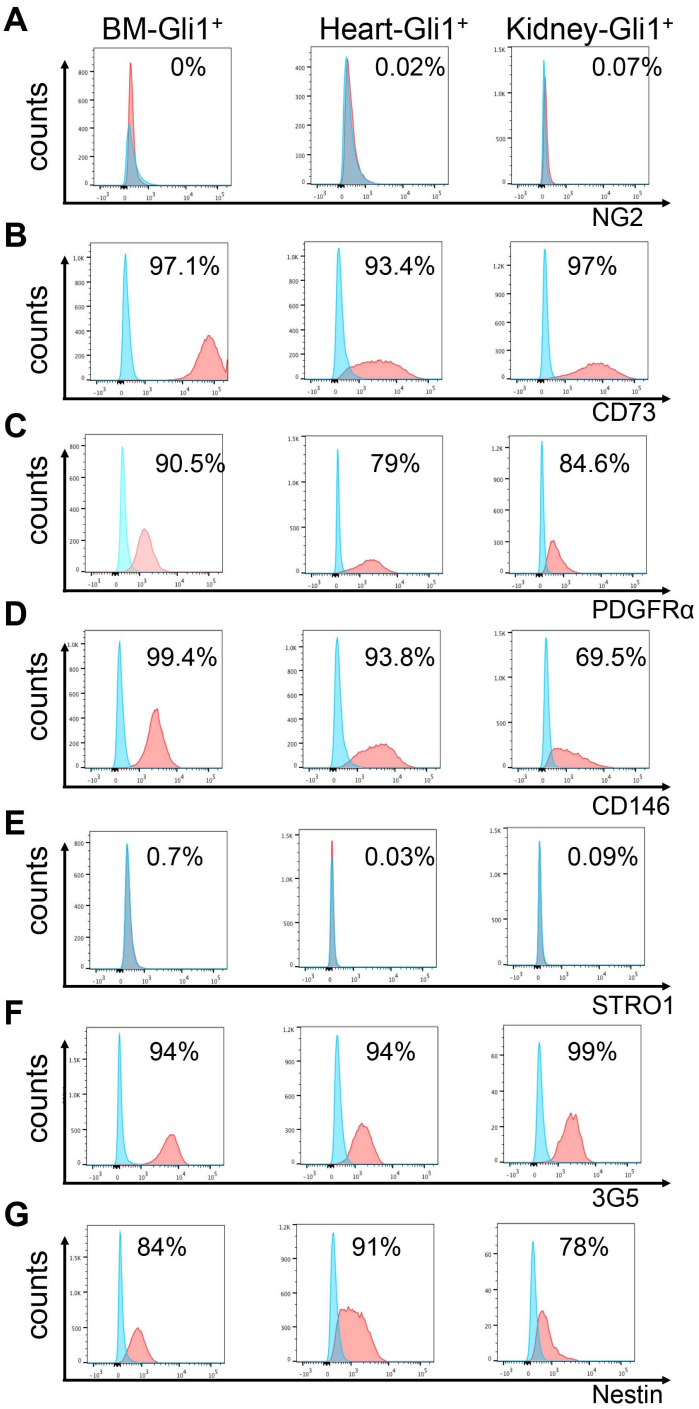

Figure S2: Related to figure 2. Representative flow cytometric plots of cultured Gli1$^+$ cells.

Cells were isolated from whole heart and kidney by digestion and from bone chips (BM), subsequently cultivatied, fluorescence activated cell sorted and additionally cultured for 8 weeks prior to flow cytometric characterization. Representative histograms for expression of: **(A)** NG2 **(B)** CD73 **(C)** PDGFRα **(D)** CD146 **(E)** STRO1 **(F)** 3G5 and **(G)** Nestin (negative control in blue, Gli1tdTomato$^+$ cells in red).

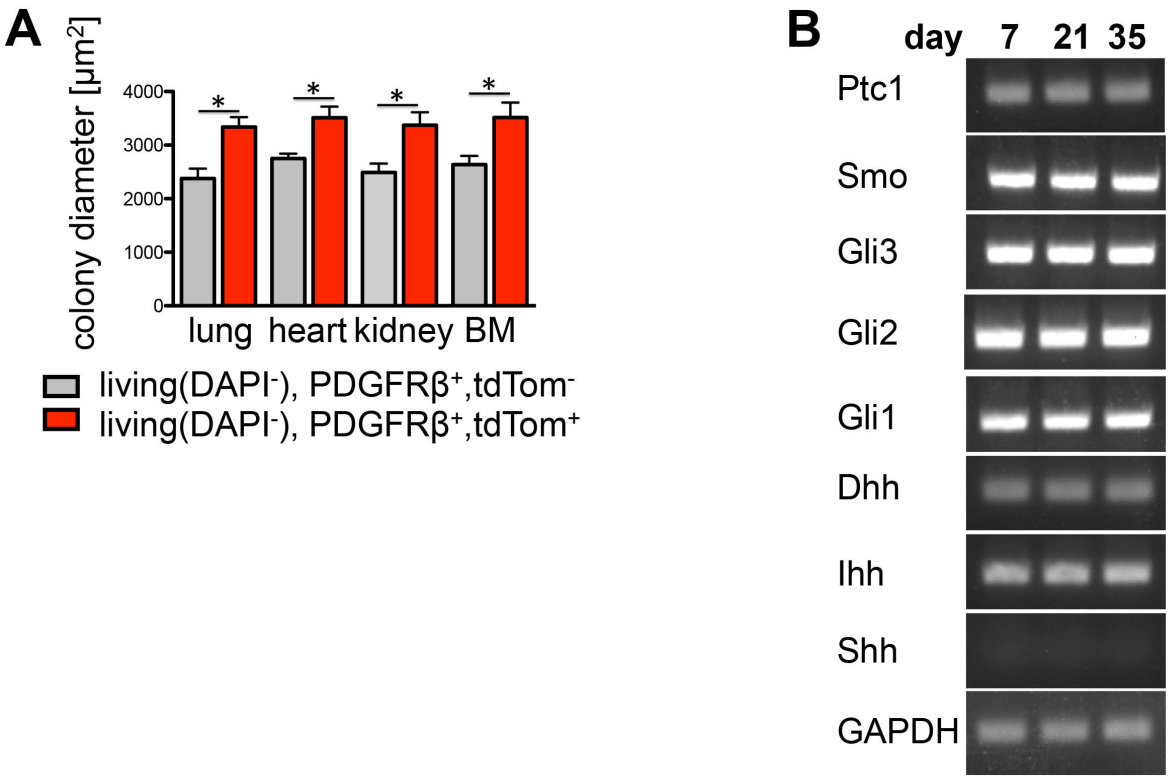

Figure S3: Related to figure 3. Colony size and mRNA expression of hedgehog pathway members in Gli1$^+$ cells in vitro.

(A) The size of the formed colonies at 7 days was assessed by measurement of the colony diameter using inverted microscopy from fluorescence activated cell sorted, living (DAPI$^-$), PDGFRβ$^+$,tdTomato(tdTom)$^-$ and PDGFRβ$^+$,tdTomato$^+$ cells from whole digested lung, heart, kidney and bone marrow (bone chips isolation method) in 6 wells (n=3 biologic replicates each) *p<0.05 by t-test.

(B) Representative PCR bands of the hedgehog pathway members patched 1 (Ptc1), smoothened (Smo), Gli1-3, desert hedgehog (Dhh), Indian hedgehog (Ihh) and sonic hedgehog (Shh) after 7, 21 and 35 days of culture from fluorescence activated cell sorted Gli1$^+$ cells liberated from bone chips.

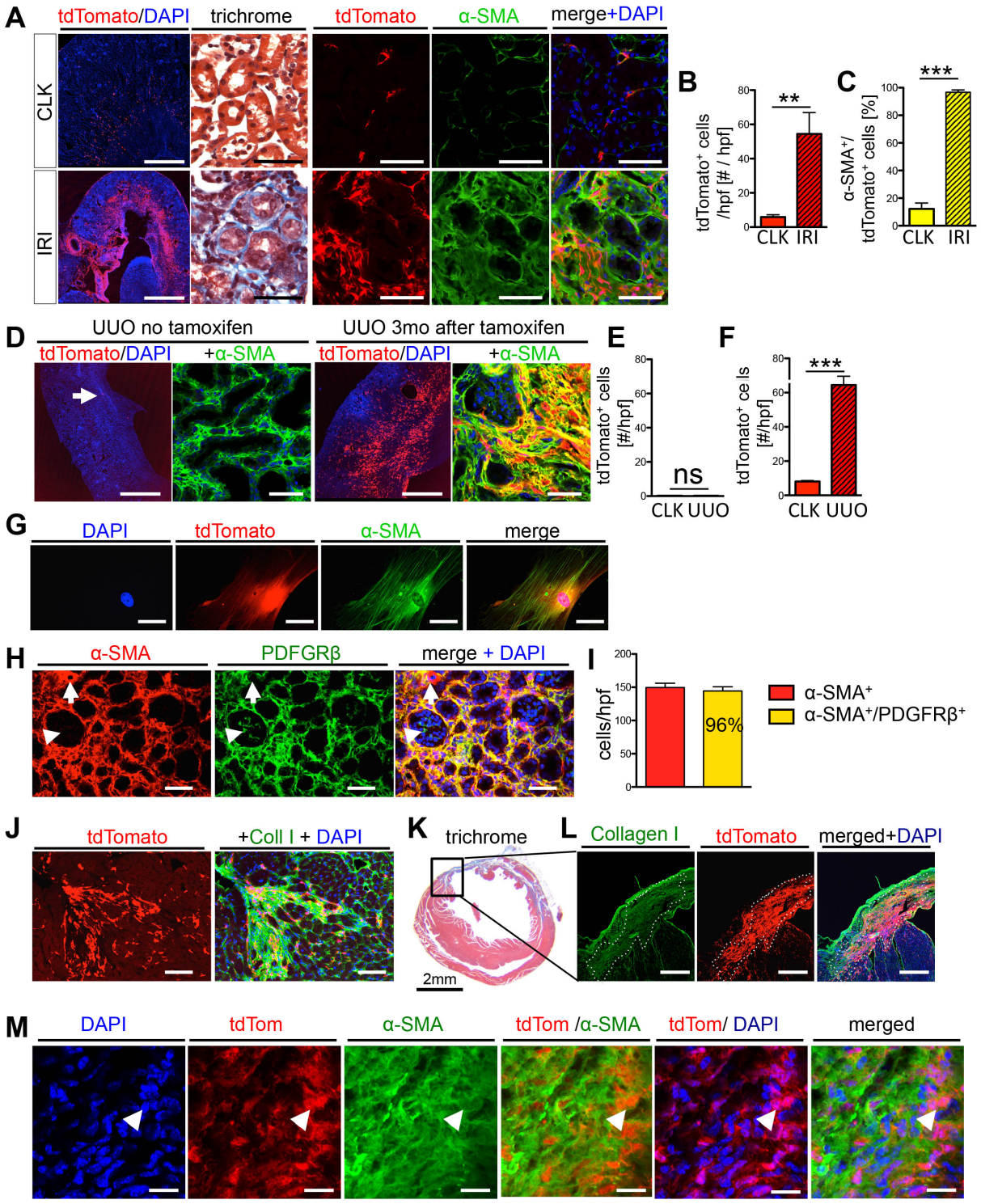

Figure S4: Related to figure 4. Fate tracing of Gli1$^+$ cells in kidney and heart fibrosis *in vivo* and validation of the genetic mouse fate tracing approach.

(A-C) Bigenic Gli1,tdTomato$^+$ mice were injected with tamoxifen, underwent unilateral ischemia reperfusion injury (IRI) of the kidney 10 days after the last injection of tamoxifen and were euthanized 4 weeks after the surgery (CLK contralateral non-injured kidney).
Following severe IRI, the Gli1-tdTomato$^+$ cells dramatically expanded and acquired alpha smooth muscle actin (α-SMA) expression during progression of kidney fibrosis. Scale bars: left panel 500µm, all others 50µm, **p<0.01, ***p<0.001 by t-test, data is presented as mean±SEM, high power field (hpf) 400x

(D-F) To exclude leaky recombination in the absence of tamoxifen or residual tamoxifen in the system which might drive recombination of cells inducing Gli1$^+$ expression following injury, we performed two mouse UUO experiments in bigenic Gli1CreER^{t2},tdTomato mice. A group of 3 bigenic mice underwent UUO surgery without tamoxifen treatment and sacrificed 10 days later, and another group of n=3

bigenic mice underwent UUO surgery 3 months after receiving tamoxifen, to exclude remaining tamoxifen in the mouse-system during injury. There was no significant recombination in the groupt that did not receive tamoxifen (arrow: one tdTomato$^+$ cell) even during severe fibrosis following UUO with interstitial (α-SMA$^+$) myofibroblasts. However, in the second group, where UUO surgery was performed late (3 months) after tamoxifen injection, we observed a significant expansion of tdTomato$^+$ cells with a comparable fold change increase between contralateral (CLK) and UUO kidneys as in our initial experiments with only 10 days between tamoxifen administration and surgery. These results excluded the possibility that remaining tamoxifen would have resulted in recombination in cells that acquired Gli1 expression following injury or during disease progression. Scale bars 500μm left panels and 50μm right panels. ***p<0.001 by t-test

(G) Gli1-tdTomato$^+$ sorted (FACS) cells from the heart treated with TGF-β for 24h and stained for α-SMA. tdTomato intensity is highest in the nucleus with a lower signal in the cytoplasm whereas for α-SMA a converse distribution can be observed, with no signal in the nucleus and α-SMA$^+$ stress-fibers around the nucleus and in the cytoplasm. Therefore, to assess co-localization of tdTomato and α-SMA the perinuclear region has to be evaluated. Scale bars 50μm

(H-I) 10 day UUO kidneys co-stained for α-SMA and PDGFRβ indicating that the vast majority of kidney myofibroblasts (α-SMA$^+$) expresses PDGFRβ (Quantification in n=3 wild-type mice with n=4 random high power fields-400x from inner cortex and outer medulla each). Scale bars 50μm

(J) Large interstitial myocardial scar 8 weeks after ascending aortic constriction (AAC) in bigenic Gli1,tdTomato$^+$ mice. Scale bars: 100μm

(K-M) Bigenic Gli1,tdTomato$^+$ mice received tamoxifen injection (3x0.1mg/kg bodyweight i.p.), underwent left coronary artery ligation surgery (left anterior descending, LAD) 10 days after the last tamoxifen injection and were sacrificed 28 days after surgery. Representative picture of a trichrome stained heart 4 weeks after coronary artery ligation (K) shows the infarction scar of the left ventricular wall. Collagen I immunostaining was used to delineate the scar area of the left ventricle (dashed line in L). Gli1$^+$ cells expanded within the scar. Co-staining revealed that Gli1,tdTomato$^+$ cells acquired perinuclear (arrowheads in M) alpha smooth muscle (α-SMA) expression. Scale bars 500μm in L and 20μm in M.

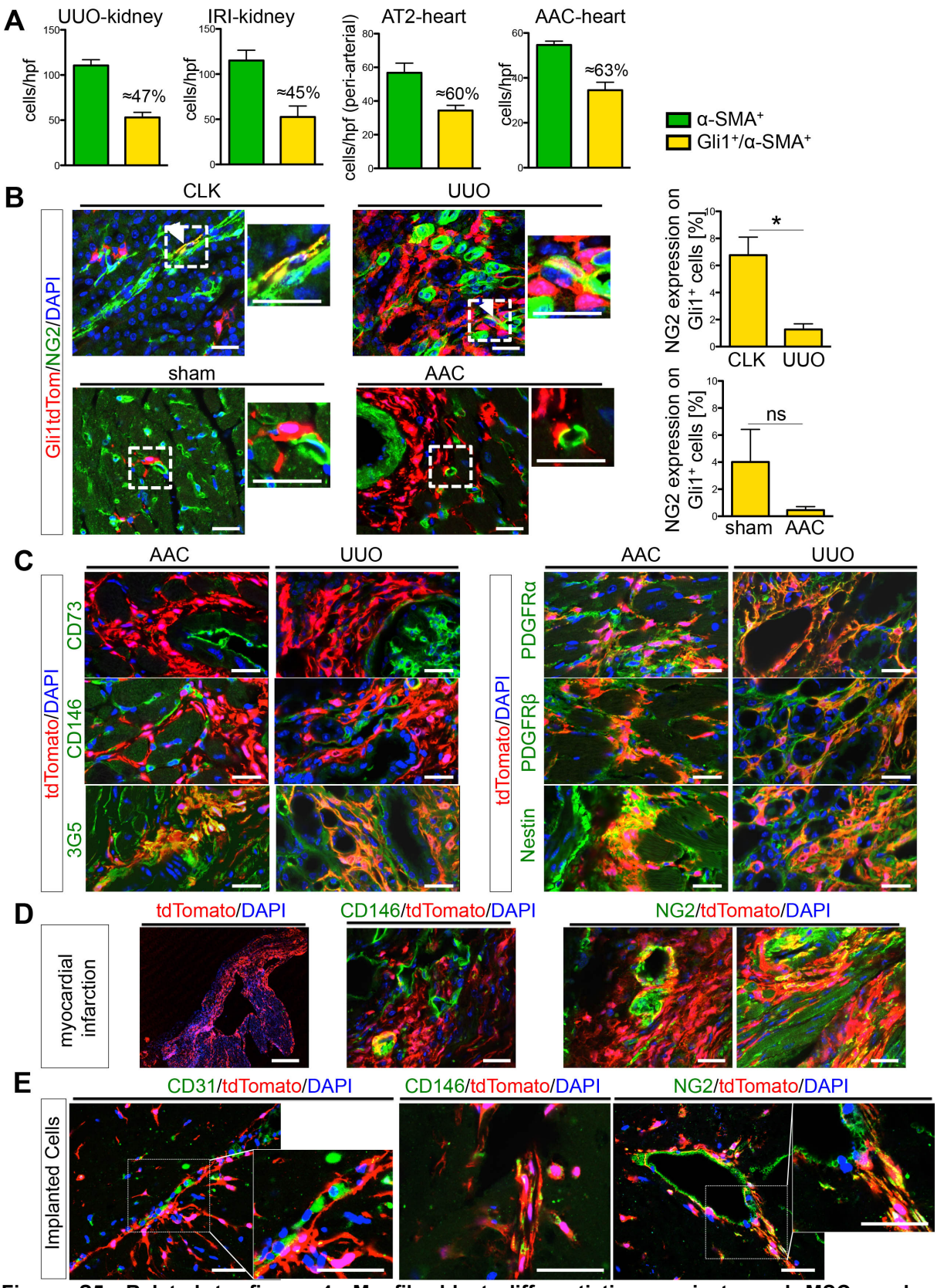

Figure S5: Related to figure 4. Myofibroblast differentiation, pericyte and MSC marker expression of Gli1$^+$ cells in kidney and heart fibrosis and fate tracing of Gli1$^+$ cells angiogenesis in vivo.

(A) Quantification of all interstitial α-SMA$^+$ cells and the number of α-SMA$^+$ cells that co-label with tdTomato in kidney fibrosis (unilateral ureteral obstruction-UUO and ischemia reperfusion injury-IRI) and heart fibrosis (angiotensin 2 – AT2 and ascending aortic constriction-AAC). Quantification was performed by cell-counting in 5-high power fields (hpf 400x) from n=4 mice in UUO and n=5 mice in

IRI, n=4 mice in AT2 vs n=3 mice with vehicle mini-pump, n=4 mice with AAC and n=3 sham AAC mice.

(B) NG2 expression by immunostaining in kidneys (CLK and UUO) and heart (sham versus ascending aortic constriction-AAC) from bigenic Gli1CreER^{t2},tdTomato mice (arrowheads indicate co-labeling). Quantification by cell counting in 3 high power fields-hpf 400x of n=4 mice in UUO and CLK, n=4 AAC and n=3 sham-AAC mice) *$p<0.05$ by t-test Scale bars 25μm

(C) Immunostained fibrotic heart (ascending aortic constriction-AAC) and kidneys (unilateral ureteral obstruction-UUO) for the pericyte and/or mesenchymal stem cell markers CD73, CD146, 3G5, PDGFRα, PDGFRβ and Nestin. Scale bars 25μm

(D) Left ventricular scar 4 weeks after coronary artery ligation in a bigenic Gli1CreER^{t2},tdTomato mouse, costaining with the pericyte markers CD146 or NG2. Scale bars left panel 500μm all others 25μm

(E) Matrigel plugs with Gli1^{+} cells, 4 weeks after implantation in the *in vivo* angiogenesis model. Co-staining for the endothelial cell marker CD31 indicates sprouting endothelial cells with Gli1^{+} cells associated in a pericyte-like distribution around CD31^{+} endothelial cells. NG2 and CD146 co-staining indicates that Gli1^{+} cells acquire expression of these mature pericyte markers during angiogenesis. Scale bars 50μm

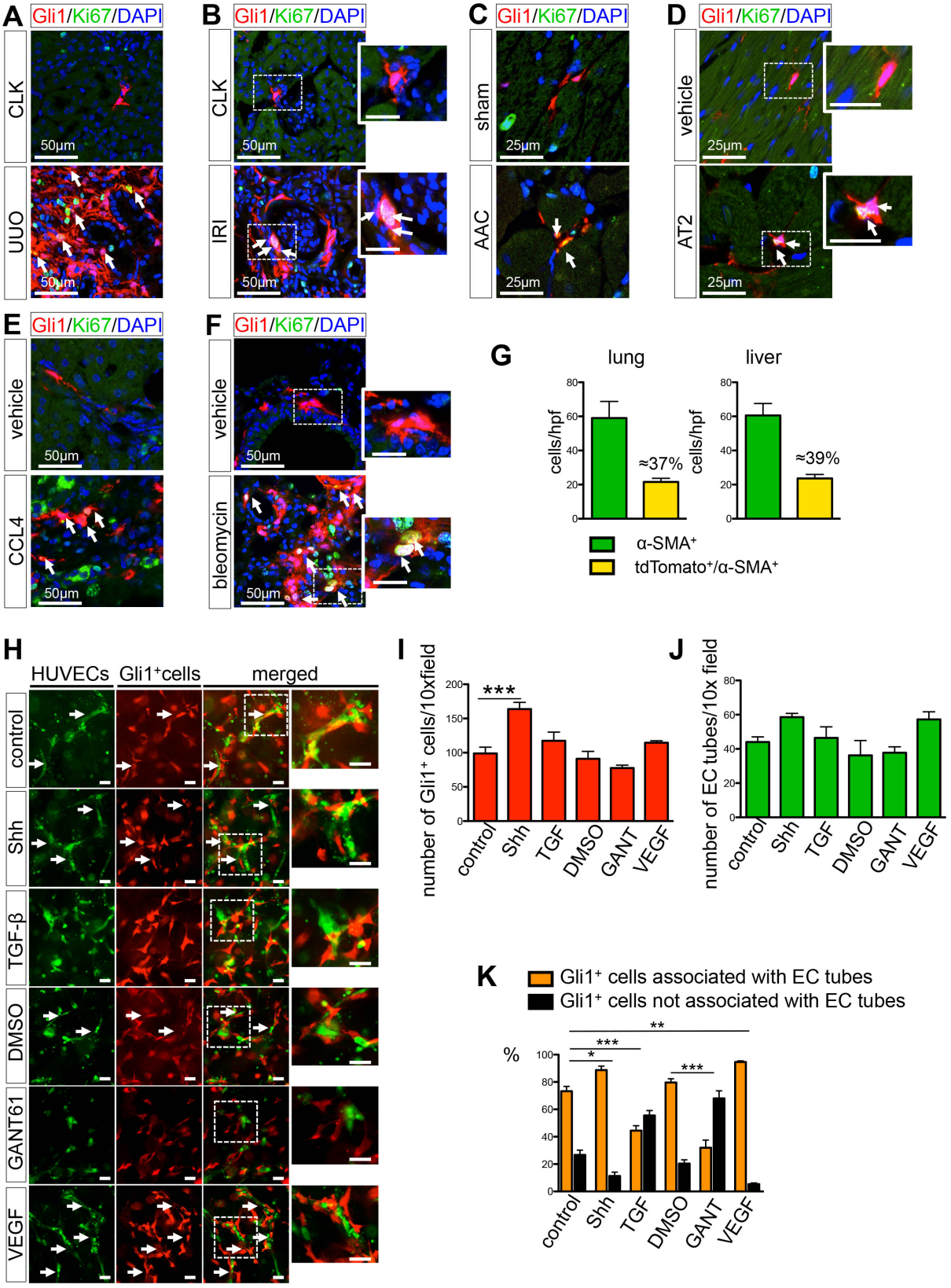

Figure S6: Related to figures 4-5. Proliferation, myofibroblast differentiation and tube formation of Gli1$^+$ cells

(A-B) Representative images of proliferating (Ki67$^+$, arrows) Gli1-tdTomato$^+$ cells following injury of the kidney (UUO unilateral ureteral obstruction, IRI unilateral ischemia reperfusion injury, CLK non-injured contralateral kidney). Scale bars: as indicated, inserts 20µm

(C-D) Representative images of proliferating (Ki67$^+$, arrows) Gli1-tdTomato$^+$ cells following injury of heart (AAC, ascending aortic constriction; AT2 angiotensin minipump; vehicle, normal saline minipump). Scale bars: as indicated, inserts 20µm

(E-F) Representative images of proliferating ($Ki67^+$, arrows) Gli1-$tdTomato^+$ cells following injury of the liver and lung (CCL4, carbon tetrachloride; vehicle-liver: corn oil; vehicle lung: normal saline). Scale bars: as indicated, inserts 20μm
(G) Quantification of all interstitial α-SMA^+ cells and the number of α-SMA^+ cells that co-label with tdTomato in lung and liver fibrosis. Quantification was performed by cell-counting in 5-high power fields (hpf 400x) from n=4 mice that received bleomycin intratracheally vs. n=3 that received vehicle intratracheally and n=5 mice that received carbon tetrachloride for liver injury intraperitoneally versus n=3 that received vehicle.
(H-K) Tube formation assay of human umbilical cord endothelial cells (HUVECs) labeled with a green cell tracer with $Gli1^+$ cells (red tdTomato protein) isolated from bone chips in 3 dimensional collagen gels *in vitro*. Representative pictures and quantification of $Gli1^+$ cell number (I), number of endothelial (EC) tubes, and $Gli1^+$ cells associated with EC tubes (K) after treatment with phosphate bufferes saline (PBS, control), sonic hedgehog, transforming growth factor β (TGF- β), GANT61 (vehicle DMSO), and vascular endothelial growth factor A (VEGF). Scale bars 100μm *$p<0.05$, **$p<0.01$, ***$p<0.001$ by one way ANOVA.

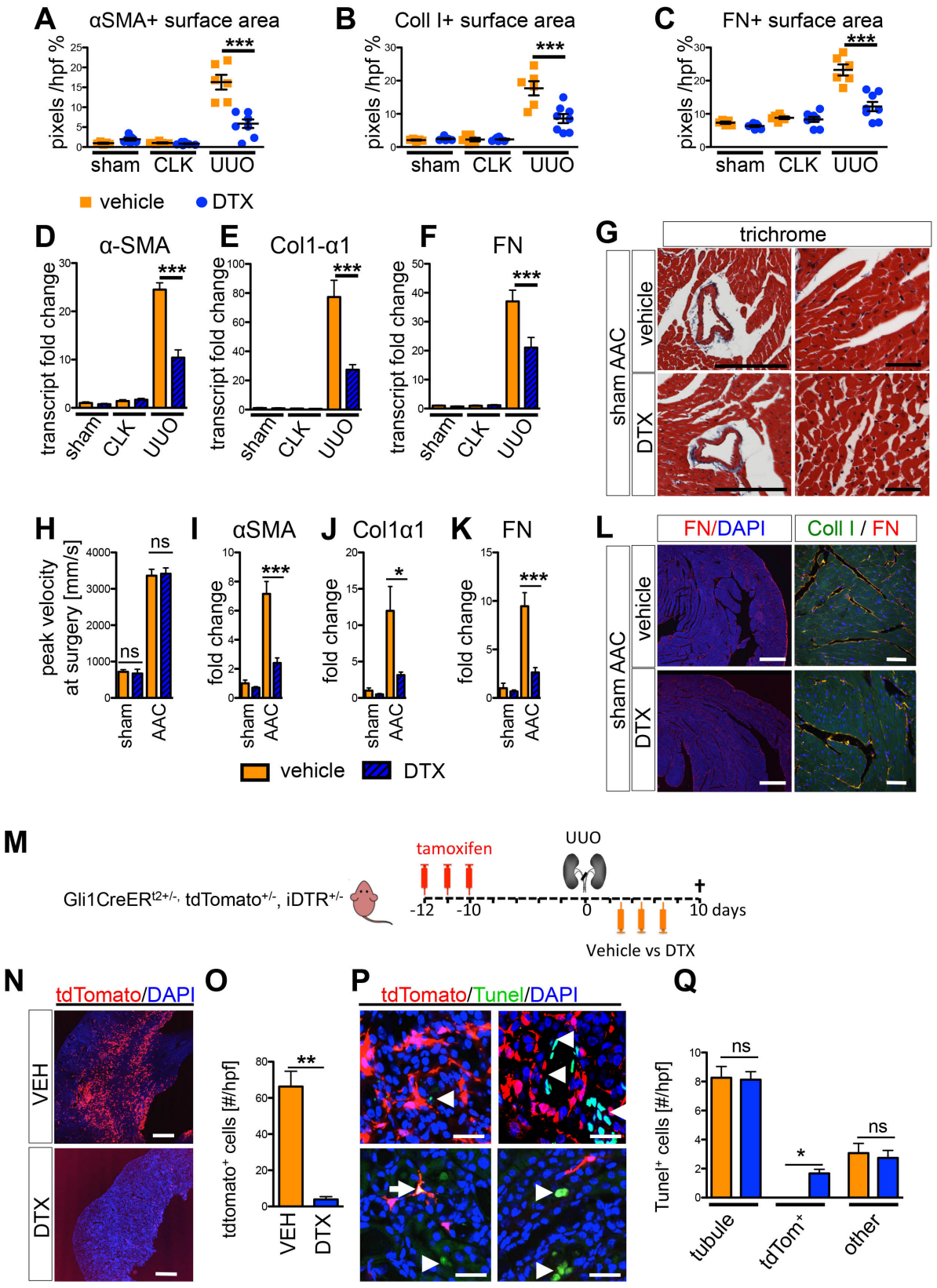

Figure S7: Related to figure 7. Genetic ablation of Gli1$^+$ cells in kidney and heart fibrosis.

(A-C) Ablation of Gli1$^+$ cells in kidney fibrosis of bigenic Gli1CreER^{t2},iDTR mice (see Figure 7 B-E). Quantification of the immunostained (A) α-SMA$^+$ surface area (B) collagen-I$^+$ surface area and (C) fibronectin$^+$ surface area (FN$^+$) in sham, contralateral (CLK) and unilateral ureteral obstruction (UUO) kidneys after injection of vehicle or diphtheria-toxin (DTX). high power field (hpf); mean±SEM; ***p<0.001 one way ANOVA with posthoc Tukey.

(B-F) Quantitative realtime PCR for mRNA expression of (D) α-SMA, (E) collagen-IαI and (F) fibronectin (FN) from whole kidney lysates of sham, CLK and UUO kidneys after injection of vehicle or diphtheria-toxin (DTX). mean±SEM; ***p<0.001 one way ANOVA with posthoc Tukey
(G-L) Ablation of $Gli1^+$ cells following ascending aortic constriction (AAC) surgery of bigenic $Gli1CreER^{t2}$,iDTR mice (see Figure 7 G-R). (G) Representative trichrome pictures of the sham group after injection with diphtheria toxin (DTX) or vehicle. (H) Echocardiographically determined peak velocity at the suture site immediately after ascending aortic constriction (AAC) or sham surgery (AAC-vehicle n=10, AAC-DTX n=9, sham-vehicle n=5, sham-DTX n=5). (I-K) Quantitative realtime PCR for mRNA expression of (I) α-SMA, (J) collagenIαI and (K) fibronectin (FN) from whole kidney lysate of sham, CLK and UUO kidneys after injection of vehicle or diphtheria-toxin (DTX). (L) Representative pictures of heart sections from sham mice that received either vehicle or DTX stained for fibronectin (FN) and collagen I (Coll I). *p<0.05, ***p<0.001 one way ANOVA with posthoc Tukey.
(N-Q) To verify a significant cell ablation and exclude side effects on other non-$Gli1^+$ cell populations, we generated triple transgenic mice that express bot the human diphtheria toxin receptor (iDTR) and tdTomato in $Gli1^+$ cells. Mice received tamoxifen and were subjected to UUO surgery and sacrificed 10 days later. Representative pictures of UUO kidneys and quantification of $tdTomato^+$ cells indicate a significant ablation of $Gli1^+$ cells by DTX with only a small number of $tdTomato^+$ cells remaining (N-O). Tunel staining was performed to quantify the number of apoptotic cells. Representative pictures of Tunel stained UUO kidneys (P) and quantification of tubular cells (tubule), Gli1-$tdTomato^+$ cells and other renal cells indicate increased apoptosis in the $Gli1^+$ cell population while we did not detect an effect on apoptosis in other cell populations. mean±SEM; *p<0.05, **p<0.01 by t-test

Movie S1: Related to figure 1. Fluorescence microangiography demonstrates the perivascular localization of Gli1+ MSC in the renal cortex.

Movie S2: Related to figure 1. Fluorescence microangiography demonstrates the perivascular localization of Gli1+ MSC in the myocard.

Methods

Animals

All mouse experiments were performed according to the animal experimental guidelines issued by the Animal Care and Use Committee at Harvard University. Gli1-nLacZ (i.e. $Gli1^{tm2Alj}$/J, JAX Stock 008211) $Gli1CreER^{t2}$ (i.e. $Gli1^{tm3(re/ERT2)Alj/}$J, JAX Stock #007913), Rosa26tdTomato (i.e. B6-Cg-Gt(ROSA)26$Sort^{tm(CAG-tdTomato)Hze}$/J JAX Stock # 007909) iDTR mice (i.e. C57BL/6-Gt(ROSA)26$Sor^{tm1(HBEGF)Awai}$/J, JAX Stock # 007900), $Ptprc^a$-$Pepc^b$ mice (B6.SJL-$Ptprc^a$-$Pepc^b$/Boy, JAX Stock # 002014) were purchased from Jackson Laboratories (Bar Harbor, ME). Offspring were genotyped by PCR according to the protocol from the Jackson laboratory. For lineage tracing studies 6-7 week old mice received 3 x 0.1mg/kg bodyweight tamoxifen in corn oil / 3% ethanol (Sigma) via intraperitoneal injection 10 days before surgery or disease induction. Unilateral ureteral obstruction (UUO) surgery was performed as previously described (Fabian et al., 2012). Briefly, after flank incision the left ureter was tied off at the level of the lower pole with two 4.0 silk ties. Mice were sacrificed at day 10 after surgery. For the unilateral ischemia re-perfusion injury (IRI), mice were anesthetized with pentobarbital sodium (60 mg/kg body weight, intraperitoneally), the left kidney was exposed through flank incision and subjected to ischemia by clamping the renal pedicle with non-traumatic microaneurysm clamps (Roboz, Rockville, MD) for 35 min. Body temperatures were controlled at 36.5°C–37.5°C throughout the procedure. For the mouse model of angiotensin 2 induced myocardial fibrosis, we implanted osmotic minipumps (Alzet Model 2004) infusing angiotensin 2 (Sigma) dissolved in sterile normal saline (Baxter) at a rate of 1000ng/kg/min subcutaneously during a ketamine/xylazine (100/13 mg/kg bodyweight) anesthesia. Osmotic minipumps containing normal saline were implanted in control animals. Mice were euthanized at 28 days after surgery. Ascending aortic constriction (AAC) was performed in the Cardiovascular Facility of the Brigham and Women´s Hospital as previously described (Liao et al., 2002). Briefly, mice were anesthetized with 3% isoflurane, intubated and ventilated on a Harvard rodent respirator (Type 845, Hugo Sachs Elektronik, March, Germany). An incision was made at the third intercostal space and ribs were gently spread with a microdissecting retractor (Biomedical Research Instruments). The ascending

aorta was then isolated from the pulmonary artery approximately 3mm from the base of the heart and ligated around a blunted 27 gauge needle using 8-0 suture (Prolene, ETHICON). The ribs, chest musculature and skin was closed using 5-0 Dexon and 6-0 Prolene suture (ETHICON). Sham- operated mice underwent the same procedure without ligation of the ascending aorta. For the mouse model of myocardial infarction, mice underwent a similar procedure with sternotomy, the proximal left anterior descending artery was identified and ligated (8-0 suture, Prolene, ETHICON). For the model of bleomycin-induced lung fibrosis, mice were anesthetized with ketamine/xylazine (100/13 mg/kg bodyweight intraperitoneally) and the trachea was exposed by cervical incision. Bleomycin (Hospira) was dissolved in normal saline and intratracheally instilled via a 29 gauge needle at a dose of 2.5 U/kg bodyweight. Mice were euthanized at day 14 and lungs were perfused with Periodate Lysine-Paraformaldehyde (PLP) buffer and inflated to 25cm H2O trough the trachea. After ligation of the trachea (3-0 suture) the lungs were immersed in PLP for 2hours on ice, placed in 18% sucrose over night, and OCT embedded. Control mice underwent the same procedure but were instilled with normal saline. Liver injury and fibrosis was induced by intraperitoneal injection of carbon tetrachloride (CCL4, Sigma dissolved in mineral oil 1:3) at a dose of 1µl/g twice weekly for 4 weeks. Control mice received intraperitoneal injections of mineral oil (Sigma) alone. For all surgical procedures mice received buprenorphine (0.1mg/kg bodyweight subcutaneously) to achieve analgesia.

In vivo angiogenesis model

The *in vivo* matrigel plug angiogenesis model was performed as decribed previously (Malinda, 2009). Briefly, $Gli1^+$ cells were isolated according to the bone chip method (Zhu et al., 2010) and purified by fluorescence activated cell sorting. Matrigel (BD #354248) was thawed on ice. After trypsinization and centrifugation of the purified and cultured $Gli1^+$ cells, the cell pellet was dissolved in matrigel at a concentration of $8x10^6$ cells / ml. Wildtype C57BL6 mice (Charles river, n=6) were injected with 2 injections (200µl) at different locations in the abdominal subcutaneous adipose tissue. After 4 weeks the mice were sacrificed, the matrigel plugs were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) and OCT frozen for cryosectioning and immunostaining.

Bone marrow transplantation experiments

For transplantation experiments of *Gli1-tdTomato* cells into CD45.1 wild type microenvironment, bigenic $Gli1CreER^{t2}$; tdTomato mice (CD45.2) received tamoxifen (3x 0.4mg/kg bodyweight p.o.) every other day until 3 days prior to euthanization, the bone marrow was harvested from leg bones and pelvis via crushing and $3x10^6$ freshly isolated whole bone marrow cells were injected into the tail-vein of lethally irradiated (1050 Rads) CD45.1 positive recipient mice (B6.SJL-$Ptprc^a$-$Pepc^b$/Boy, JAX Stock # 002014). To increase the number of $Gli1^+$ cells, $2x10^6$ FACS sorted Gli1-tdTomato+ cells isolated from bone chips of bigenic $Gli1CreER^{t2}$; tdTomato mice were added to the whole bone marrow cell suspension of each recipient prior to injection. Mice underwent UUO or sham surgery 5 weeks after cell injection and were euthanized 10 days later. Whole bone marrow was isolated via crushing of leg bones, kidney and lungs were digested and processed as described in the flow cytometry section and stained using common leukocyte variant specific antibodies (CD45.1 vs CD45.2) to verify engraftment of transplanted hematopoietic cells as described below.

Parabiosis

Bigenic $Gli1CreER^{t2}$; tdTomato mice (8 week old) received tamoxifen (3x0.4mg/kg bodyweight p.o) and were conjoined to wild-type CD45.1 mice (B6.SJL-$Ptprc^a$-$Pepc^b$/Boy, JAX Stock # 002014, 8 week old) at 10 days after the last tamoxifen injection. Parabiosis was performed as previously described (Bunster and Meyer, 1933; Ruckh et al., 2012). Anesthesia was achieved by intraperitoneal injection of 100mg/kg bodyweight ketamine, 10mg/kg bodyweight xylazine and 3mg/kg bodyweight acepromacine. Buprenorphine (0.05-0.1mg/kg s.c.), meloxicam (1mg/kg s.c.) and local lidocaine (1%, s.c.) was given for analgesia. Blood chimerism was confirmed via flow cytometry of peripheral blood drawn from

the retro-orbital plexus venosus of the CD45.1^{+} parabiont (Ptprc^{a}-Pepc^{b}) 4 weeks after parabiosis surgery. After red blood cell lysis (BD Biosciences), leukocytes were stained for CD45.1 (APC-Cy7, ebioscience #25045382) and CD45.2 (FITC, ebioscience #110454) in PBS 10% FBS. After verification of cross-circulation in the peripheral blood a UUO surgery was performed at the right kidney of the CD45.1^{+} parabiont unter 3% isoflurane (ISOTHESIA, Butler Schein) anesthesia and analgesia as described in the UUO surgery above. At 10 days after the UUO surgery the mice were euthanized. Kidneys (sham = Gli1; tdTomato-mouse, CLK = contralateral of the CD45.1 parabiont, UUO= unilateral ureteral obstruction of the CD45.1 parabiont) were digested and stained for CD45.1 and CD45.2 according to the protocol in the flow cytometry section. Single-cell suspensions of spleen were prepared by pressing tissue through a cell strainer followed by red blood cell lysis.

Cell specific ablation experiments

For all ablation experiments, bigenic Gli1CreER^{t2}; iDTR mice received tamoxifen (3x 0.4mg/kg per oral gavage) 10 days before surgery and were then injected with diphtheria toxin dissolved in PBS (List Biological Laboratories) at a dose of 50ng/g bodyweight intraperitoneally as indicated (i.e. at day 3,5,7 following UUO and days 16, 18, 37, 39 following AAC surgery). Control mice received intraperitoneal injections of PBS.

In order to validate the cell-ablation, triple transgenic mice (Gli1CreER^{t2}; iDTR; tdTomato) were generated and injected with the above mentioned dose of tamoxifen. UUO surgery was performed 10 days after the last tamoxifen dose and DTX was administered in analogy to the other UUO ablation experiments. Mice were sacrificed 10 days after UUO surgery.

Tissue Preparation and Histology

Mice were anesthetized with isofluorane (Baxter) and perfused via the left ventricle with 4°C PBS for 1 minute. For histological analyses tissue sections were fixed in 10% formaldehyde for 1h, paraffin embedded and cut with a rotating microtome at 3µm thickness and stained according to routine histology protocols. For immunofluorescence studies, kidneys were fixed in 4% paraformaldehyde on ice for 1 hour, then incubated in 30% sucrose in PBS at 4°C overnight. OCT-embedded (Sakura Finetek) tissues were cryosectioned into 7 µm sections and mounted on Superfrost slides (Fisher Scientific). Sections were washed in 1X PBS, blocked in 10% normal goat serum (Vector Labs) and incubated with primary antibodies specific for Ki-67 (1:200, Vector Labs Cat. #VP-RM04), alpha SMA (1:200, Sigma, Cat No. A2547), alpha SMA-FITC (1:200, Sigma, # F3777), fibronectin (1:40, Abcam #ab23750), collagen1α1 (1:20, Southern Biotechnology #1310-01), DsRed (1:50, Clontech #632496), NG2 (1:50, Millipore #AB5320), CD146 (1:100 Abcam #ab75769), CD146-APC (1:100, Biolegend #134711), CD73 (1:5000, gift from Dr. Nicolas Picard, Institute of Anatomy University of Zurich), PDGFRα (1:50, R&D Systems #af1062), STRO1 (1:100, R&D Systems mab1038) and Nestin (1:100, Abcam #ab6142). Staining for the 3G5 antigen was performed by incubation of tissue sections with supernatant from the 3G5 hybridoma cell line (ATCC CRL-1814). Secondary antibodies were FITC-, Cy3, or Cy5-conjugated (Jackson ImmunoResearch). Sections were then stained with DAPI (4´,6´-´diamidino-2-phenylindole) and mounted in Prolong Gold (Life Technologies). Tunel staining was performed using the in situ cell death detection kit (Roche) according to the manufacturer instructions. Immunohistochemistry was performed using the primary antibody against Gli1 (1:500 R&D Systems #MAB3324) and a biotynilated secondary antibody (Jackson Immuno). Antigen retrieval was achieved by pressure cooker treatment and antigen unmasking solution (Vector). Staining was achieved using Avidin/Biotin Blocking kit, the ABC kit, the DAB kit and the DAB enhancing solution (all Vector laboratories) according to the manufacturer instructions.

For quantification of cell expansion, pictures (400x, n=5/organ) were taken as follows: in the kidney models random pictures of the inner cortex and outer medulla, in the angiotensin 2 model of myocardial fibrosis random pictures around arteries i.e. periarterial; in the AAC model random pictures of the interstitial myocardium (excluding arteries), in the liver-fibrosis model random pictures around central veins and the periportal field and in the bleomycin induced lung fibrosis model random pictures of interstitial lung (excluding large

bronchi) were taken. Positive cells were counted manually using Image J (NIH). Quantification of α-SMA, collagen I and fibronectin positive surface area was performed by taking random pictures (400x, n=5 per organ) of each mouse using the number of stained pixels per total pixels in Adobe Photoshop CS5 (Adobe Systems, Inc., San Jose, CA). All images were obtained by confocal (Nikon C1 eclipse, Nikon, Melville, NY) or standard microscopy (Nikon eclipse 90i).

Fibrosis severity was scored at 400x magnification using a counting grid with 117 intersections (for the kidney 5 random pictures of the inner cortex, for the heart 6 pictures of different left ventricular regions i.e. anterior, anteroseptal, inferoseptal, inferior, inferolateral were used). The number of grid intersections overlying trichrome positive (blue) interstitial area was counted and expressed as a percentage of all grid intersections. For this calculation in the kidney, intersections that were in tubular lumen and glomeruli were subtracted from the total number of grid intersections. Cardiomyocyte cross sectional area was determined by measuring the cell surface area of 10 random cardiomyocytes in 400x pictures of the myocardium (3 trichrome stained pictures / heart) using Image J (NIH). To identify LacZ activity in kidney sections, PFA fixed frozen sections were incubated in standard 5-bromo-4-chloro-3-indolyl-β-d-galactoside (X-gal) for 48 hours counterstained with nuclear fast red and mounted. Fluorescence microangiography was performed as recently described (Kramann et al., 2014).

Flow Cytometry and cell sorting

For flow cytometric analysis or fluorescence activated cell sorting (FACS) of whole organs and tissues, mice were euthanized exactly as described above and the organs of interest were placed in FACS Buffer (PBS, 10%FBS, 2% Penicillin Streptomycin, Life Technologies). After thoroughly mincing the tissue/organ using a sterile scalpel (Feather), the tissue/organ was placed in gentleMACS C Tubes (Miltenyi Biotec) containing 1.5ml DMEM (Life Technologies) with 0.1mg/ml Liberase TL (Roche). The tissue was then dissociated using the D program of the gentleMacs dissociator (Miltenyi Biotec) followed by 30 min incubation at 37°C. Following washing steps with FACS buffer and centrifugation (1500 rpm 5 min) the solution was filtered twice trough a 40µm cell strainer (BD Biosciences) and transferred to 5ml Polystyrene Round-Bottom FACS tubes (BD Biosciences). FACS sorting was performed using the FACSAria II cell sorter (BD Biosciences). For flow cytometric studies the samples were stained in 100-500µl FACS Buffer using the following fluorochrome conjugated antibodies: CD31-APC (Biolegend #102410), CD45-FITC (eBioscience #17-0291-82), CD34-FITC (eBioscience #11-0341-85), CD29-APC (eBioscience #17-0291-82), Sca1-APC-Cy7 (Biolegend #108126), CD44-PE-Cy7 (eBioscience #25-0441-82), CD105-PE-Cy7 (Biolegend #120410), PDGFRβ (CD140b-APC Biolegend #136008), CD45.1 (APC-Cy7, ebioscience #25045382), CD45.2 (FITC, ebioscience #110454), CD146-APC (Biolegend # 134711), CD73-APC (Biolegend 127209) all 1:100 for 30 min, or the following primary non-conjugated antibodies NG2 (1:50, Millipore #AB5320), CD146 (1:100 Abcam #ab75769), PDGFRα (1:50, R&D Systems #af1062), STRO1 (1:100, R&D Systems mab1038), Nestin (1:100, Abcam #ab6142) for 30min. 3G5 staining was achieved by incubation of cells in supernatant of the 3G5 hybridoma cell line (ATCC #CRL-1814). The staining of the intracellular antigens Nestin was performed after fixation and permeabilization of the cells (Cytofix/Cytoperm solution, BD Biosciences). After incubation with directly conjugated antibodies, cells were PBS washed and subjected to flow cytometry. Following incubation with primary antibodies, cells were PBS washed and incubated with the appropriate AF647-conjugated antibody (Jackson Immuno). All flow cytometric analyses were performed at a LSR II Flow Cytometer (BD Biosciences). For all flow cytometric analyses and sorting DAPI (1mg/ml 1:1000) was added in order to exclude dead cells. Data were analyzed by using Flow Jo software (Version 9.6.2, Tree Star Inc).

Cell Culture Experiments

$Gli1^+$ cells were grown in alpha MEM (GlutaMAX, Life Technologies) containing 20% MSC qualified FBS (Life Technologies), 2% Penicillin Streptomycin (Life Technologies),

1ng/ml murine basic fibroblast growth factor (Thermo Fisher Scientific) and 5ng/ml murine epidermal growth factor (Peprotech). Bone marrow MSC were isolated from compact bone chips of femur, tibia and pelvis according to the protocol by Zhu et al. (Zhu et al., 2010). For osteogenic or adipogenic differentiation flow-purified cells were plated in a 48 well, at a 60-70% or 90-100% confluence, the alpha MEM medium was exchanged with osteogenic or adipogenic differentiation medium, respectively (R&D Systems). After 21 days of cultivation, cells were stained according to routine protocols using Oilred O (Sigma). To achieve co-staining for alkaline phosphatase and von Kossa staining, cells were washed once with PBS, fixed with 10% neutral formalin buffer (VWR) for 10min, incubated for 15 min with distilled water followed by an incubation in an Naphthol-AS MX-PO4 (Sigma), Dimethylformamide (Fisher), Tris-HCL buffer with Red Violet LB salt (Sigma) for 45 min. After washing with distilled water cells were stained with 2.5% silver nitrate (Sigma) for 30 minutes.

For chondrogenic differentiation 2-10x10^4 cells were resuspended in 1ml chondrogenic differentiation medium (RnD Systems) in a 15ml conical tube (BD Biosciences) and centrifuged at 1500rpm for 5 min. The cell-pellets were cultivated upright in the 15 ml conical for 21 days. For detection of chondrogenic differentiation the cell-pellet was fixed in 4% paraformaldehyde. OCT-embedded (Sakura Finetek) cell-pellets were cryosectioned into 4 µm sections and mounted on Superfrost slides. After PBS washing the sections were stained with Alcian blue (Santa Cruz Biotechnology) and counterstained with nuclear fast red (Sigma). For cell culture experiments, FACS sorted tdTomato+ cells from heart or kidney were cultured in 6 well plates, serum starved overnight (0.5% FBS) and treated with 10ng/ml TGF-β or vehicle for 24 hours. For α-SMA staining cells were grown on coverslips (Fisher) coated with collagen I (BD Biosciences) and treated with TGF-β for 72 hours. Sonic hedgehog conditioned media was produced from supernatant of confluent Cos7 cells stably transfected with pcDNA3-N-Shh or pcDNA3 control plasmid. In order to study Gli1 protein expression, FACS purified Gli1$^+$ cells from bone chips were serum starved for 24h (0.5% FBS) and cultured in MesenCult MSC medium (STEMCELL Technologies) containing 20% control supernatant with either PBS, 5µM cyclopamine (Sigma, 25mM stock in ethanol), 5µM GANT61 in DMSO (Cayman Chemical), DMSO (vehicle control for GANT61) or with 400µl of supernatant of Cos7 cells stably transfected with pcDNA3-N-Shh with our without addition of cyclopamine. Equivalent concentrations of ethanol (1:5000) were added to all conditions to control for the cyclopamine solvent. Medium was changed after 24h and cells were harvested for western blot after a total of 48h

CFU-F assay

Adult 8 week old bigenic Gli1CreER^{t2}; tdTomato mice received tamoxifen (3x0.4mg/kg BW, p.o.) every other day and were euthanized 3 days after the last tamoxifen injection. Whole organs were digested and stained for PDGFRβ (CD140b-APC, biolegend) as described above. PDGFRβ$^+$,tdTomato- or PDGFRβ+,tdTomato+ cells were sorted into 6 well plates using the FACSAria II cell sorter (BD Biosciences) in densities of 10^2 ~ 10^4 cells in MesenCult MSC medium (STEMCELL Technologies) for 14 days as previously described (Park et al., 2012). The presence of more than 50 cells in a cluster at 14 days after FACS was counted as a colony. For CFU-F assay and manipulation of the Hh pathway cells were sorted in 1.6ml MesenCult MSC medium in 6 wells containing Cos7 control supernatant (0.4ml) and addition of either PBS, 5µM cyclopamine (Sigma), 5µM GANT61 in DMSO (Cayman Chemical), DMSO (vehicle control for GANT61) or with 400µl of supernatant of Cos7 cells stably transfected with pcDNA3-N-Shh. Equivalent concentrations of ethanol (1:5000) were added to all conditions to control for the cyclopamine solvent. Medium was changed daily starting 48h after sort. Colonies were quantified at 14 days as mentioned above.

In vitro tube formation assay

Human umbilical cord endothelial cells (HUVECs, ATCC) were cultured in endothelial cell medium (EGM-2 Lonza) and propagated at 80% confluency. Gli1$^+$ cells were isolated from bone chips, purified via FACS and cultured as previously described. The tube formation assay was performed as follows: HUVECs (passage 5) were fluorescence labeled (CellTracker Green, Life Technologies) 1h prior to trypsinization according to the

manufacturer's instructions. Labeled HUVECs and $Gli1^+$ cells (passage 4) were trypsinized and resuspended at a concentration of $1x10^7$cells/ml in 1xDMEM (Gibco). A collagen solution was prepared as previously described (Koh et al., 2008), 40µl of $Gli1^+$ cells and 200µl of HUVECs were added to the collagen solution. The cell collagen solution was then gently mixed and 28µl were plated in 96 half-area well TC-treated microplates (Corning). The plate was incubated for 15min at 37°C ($5\%CO^2$) to allow for collagen polymerization. Afterwards 100µl EGM-2 medium (without VEGF) was added containing either 20% control supernatant of Cos7 cells or Shh supernatant. TGF-β (10ng/ml, Peprotech), GANT61 in DMSO (5µM), DMSO, or vascular endothelial growth factor (20ng/ml VEGF-165, Peprotech) was added, respectively. Medium was changed after 24h. The total number of EC tubes, and $Gli1^+$ cells and the number of $Gli1^+$ cells adjacent to EC tubes was quantified by counting in 10x pictures using inverted fluorescence microscopy at 72h.

Immunoelectron Microscopy

For immunoelectron microscopy, kidney tissue was fixed in Karnovsky's fixative and processed using standard electron microscopy procedures. After processing, ultrathin sections were obtained and antigen retrieval was performed in 0.05M Tris buffer (Tris, pH 10.0) for 24 hours at 65°C. Following antigen retrieval, grid-mounted tissue sections were immersed in a blocking solution (0.5% goat serum and 0.05% bovine serum albumin in 0.02M phosphate buffer), washed 3 times, then incubated with a primary antibody against tdTomato (1:10, Clontech #6324961) overnight at 4 degrees. This was followed by 3 washing steps and incubation with 20nm gold particle-labeled goat secondary antibody specific for rabbit IgG (1:10, GAR20; BBInternational, Cardiff, UK) at room temperature for 1 hour. Images were obtained using the JEOL JEM-1011 transmission electron microscope after staining the tissue with uranyl acetate and lead citrate

Real Time PCR Experiments

Tissue or cell-pellets were harvested and immediately snap frozen in liquid nitrogen. RNA from kidneys or cell-culture was extracted according to the manufacturer instructions using the RNeasy Mini Kit (Qiagen) and 600 ng of total RNA was reverse transcribed with iScript (BioRad). RNA from hearts (left ventricle) was isolated using Trizol Reagent (Life Technologies) according the manufacturer instructions. Following or during the RNA extraction DNA was removed by a DNAse digestion step (Life Technologies). Quantitative polymerase chain reactions were carried out with iQ-SYBR Green supermix (BioRad) and the BioRad CFX96 Real Time System with the C1000 Touch Thermal Cycler. Cycling conditions were 95°C for 3 minutes then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, followed by one cycle of 95°C for 10 seconds. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. Data was analyzed using the $2^{-\Delta\Delta ct}$ method. PCR products were electrophoresed on a 2% agarose gel in 1xTE and visualizes under UV light with ethidium bromide. Primers are listed in table S1.

Western Blot

Tissue was snap frozen in liquid nitrogen immediately after mice were euthanized and stored at -80°C. Kidney tissue samples were homogenized in lysis buffer containing 10mM HEPES, pH 7.4, 0.32M sucrose, 2mM EDTA, 1mM DTT, 1mM PMSF and 1 protease inhibitor tablet per 10ml of lysis buffer (Roche Cat. No. 11836153001). Protein from $Gli1^+$ cultured cells was isolated after PBS washing using a cell scratcher with the same buffer. Protein from heart tissue samples (left ventricles) was isolated via precipitation from the Trizol Reagent as described elsewhere.(Simoes et al., 2013). The samples were sonicated and protein concentration was determined by the Bradford Assay using Bio-Rad Protein Assay Dye (Biorad). 10-20µg of protein from lysates was loaded on a 10% polyacrylamide gel and separated by SDS electrophoresis. Proteins were transferred to a Immobilion membrane (Millipore) blocked in 5% milk in PBST, probed overnight at 4°C with the primary antibodies: mouse anti-αSMA at 1:4000 (Sigma #A2547), rat anti-Gli1 at 1:1000 (R&D Systems #MAB3324, and rabbit anti-GAPDH at 1:4000 (Bethyl Laboratories, #A300-641A). Following incubation with primary antibody blots were washed probed with respective horseradish-peroxidase conjugated secondary antibodies at 1:4000 (Dako, Cat. No. P0447,

P0448, P0450) for 1 hour at room temperature and then visualized using the Western Lightning ECL kit from PerkinElmer (#NEL100001EA).

Echocardiography

Echocardiography was performed in the AAC cell-ablation experiment immediately following sham or AAC surgery to determine the peak velocity at the ascending aorta or the constriction, 2 weeks after surgery before the randomization to DTX or Vehicle group and at 4 and 8 weeks after the surgery in low dose isoflurane anesthesia (1.5%) using a 28MHz linear array transducer connected to a digital ultrasound console (Vevo 2100 Visualsonics). Mice were gently restrained on a heated platform and pre-warmed ultrasound gel was used on their depilated chest. B-Mode loops were acquired from parasternal short and long-axis views and M-Mode images were recorded from parasternal short-axis view at the mid-papillary level. Peak velocity at the suture was determined by pulse waved (PW) Duplex sonography of the ascending aorta using color duplex sonography as a guide for detecting the area of strongest turbulence. Images and loops were stored and analyzed offline using the Vevo 2100 analysis software (1.6.0 Visualsonics). Left ventricular ejection fraction (EF) was calculated by identification of frames with the maximal and minimal cross-sectional area and width in the parasternal long axis view as described before (Zhang et al., 2007).

Statistical Analysis

Data are presented as mean±SEM . Comparison of two groups was performed using unpaired t-test. Paired t-test was used for comparsion of repeated measured in the same group. For multiple group comparison analysis of variance with posthoc Tukey correction was applied. Statistical analyses were performed using GraphPad Prism 5.0c (GraphPad Software Inc., San Diego, CA). A p value of less than 0.05 was considered significant.

Primer pairs used for PCR

Gene	Sequence
GAPDH	Fw 5´-AGGTCGGTGTGAACGGATTTG -3´
	Rv 5`-TGTAGACCATGTAGTTGAGGTCA -3´
iDTR (HB-EGF)	Fw 5´-GGAGCACGGGAAAAGAAAG-3´
	Rv5´-GAGCCCGGAGCTCCTTCACA-3
Col1α1	Fw5´- TGACTGGAAGAGCGGAGAGT-3´
	Rv5´-GTTCGGGCTGATGTACCAGT -3´
fibronectin	Fw5´-ATCTGGACCCCTCCTGATAGT -3´
	Rv5´-GCCCAGTGATTTCAGCAAAGG-3´
α-SMA	Fw5´-CTGACAGAGGCACCACTGAA -3´
	Rv5´- CATCTCCAGAGTCCAGCACA-3´
Gli1	Fw5´- ATCACCTGTTGGGGATGCTGGAT-3´
	Rv5´- CGTGAATAGGACTTCCGACAG -3´
Gli2	Fw5´-GTTCCAAGGCCTACTCTCGCCTG -3´
	Rv5´- CTTGAGCAGTGGAGCACGGACAT-3´
Gli3	Fw5´-AGCAACCAGGAGCCTGAAGTCAT -3´
	Rv5´- GTCTTGAGTAGGCTTTTGTGCAA-3´
Ptc1	Fw5´- GCTGGAGGAGAACAAGCAAC-3´

	Rv5´- GAGCAAACATGTGCTCCAGA -3´
Smo	Fw5´- TGCCACCAGAAGAACAAGCCA-3´
	Rv5´-CCTCCATTAGGTTAGTGCGG -3´
Shh	Fw5´-TCACAAGAAACTCCGAACGATTT -3´
	Rv5´- GATGTCCACTGCTCGACCC-3´
Ihh	Fw5´-CTCTTGCCTACAAGCAGTTCA -3´
	Rv5´- CCGTGTTCTCCTCGTCCTT-3´
Dhh	Fw5´-GCCATCGCGGTGATGAACA -3´
	Rv5´-GCCTTCGTAGTGGAGTGAATCC -3´

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